Advancements in DNA vaccine vectors, non-mechanical delivery methods, and molecular adjuvants to increase immunogenicity

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Abstract

A major advantage of DNA vaccination is the ability to induce both humoral and cellular immune responses. DNA vaccines are currently used in veterinary medicine, but have not achieved widespread acceptance for use in humans due to their low immunogenicity in early clinical studies. However, recent clinical data have re-established the value of DNA vaccines, particularly in priming high-level antigen-specific antibody responses. Several approaches have been investigated for improving DNA vaccine efficacy, including advancements in DNA vaccine vector design, the inclusion of genetically engineered cytokine adjuvants, and novel non-mechanical delivery methods. These strategies have shown promise, resulting in augmented adaptive immune responses in not only mice, but also in large animal models. Here, we review advancements in each of these areas that show promise for increasing the immunogenicity of DNA vaccines.

Introduction

The constant emergence, and re-emergence, of known and novel pathogens challenges researchers to develop new vaccination technologies that allow for the rapid development of safe and effective vaccines. Nucleic acid (DNA and RNA) vaccines have characteristics that meet these challenges, including ease of production, scalability, consistency between lots, storage, and safety. DNA vaccine technology usually is based on bacterial plasmids that encode the polypeptide sequence of candidate antigens. The encoded antigen is expressed under a strong eukaryotic promoter, yielding high levels of transgene expression.[1] Inclusion of transcriptional enhancers, such as Intron A, enhance the rate of polyadenylation and nuclear transport of messenger RNA (mRNA).[2] The vaccine plasmids are generally produced in bacterial culture, purified, and then used to inoculate the host.

Modern DNA vaccine design generally relies on synthesis of the nucleic acid and possibly one-step cloning into the plasmid vector, reducing both the cost and the time to manufacture. Plasmid DNA is also extremely stable at room temperature, reducing the need for a cold chain during transportation. Vaccination with DNA plasmid removes the necessity for protein purification from infectious pathogens, improving safety. Furthermore, DNA vaccination has an excellent safety profile in the clinic, with the most common side effect being mild inflammation at the injection site.[3] Importantly, DNA vaccines provide a safe, non-live vaccine approach to inducing balanced immune responses, as the *in vivo* production of antigen allows for presentation on both class I and class II major histocompatibility complex (MHC) molecules (**Figure 1**). This elicits antigen specific antibodies [4], as well as cytotoxic T lymphocyte responses (CTL) [5], something that remains elusive in most non-live vaccines. DNA vaccines have also demonstrated

the ability to generate follicular T helper populations [6], which are critical for the induction of high quality antigen-specific B cell responses.[7]

DNA vaccination has proven successful in several animal models for preventing or treating infectious diseases, allergies, cancer, and autoimmunity.[8-12] The early success of small animal studies led to several human clinical trials. However, the protective immunity observed in small animals and non-human primates was not observed in human studies when DNA vaccines were administered alone by needle delivery. Like the more conventional protein-based vaccines, DNA can be delivered by a variety of routes, including intramuscular (IM), intradermal (ID), mucosal, or transdermal delivery. Because DNA plasmids must enter host cell nuclei in order to be transcribed into mRNA, the early failure of DNA vaccines to elicit strong responses in humans was largely due to their delivery by needle injection, which deposits the DNA in intracellular spaces, rather than within cells. Improved delivery technologies, such as intramuscular or intradermal electroporation, have been used to facilitate transport of DNA into cells, resulting in much better immunogenicity in both clinical and non-clinical studies.[13-19] In one study, electroporation-enhanced DNA vaccination resulted in increased polyfunctional antigen-specific $CD8^+$ T cells in patients receiving a HPV DNA vaccine expressing the E6 and E7 genes of HPV16 and HPV18 respectively.[20] The majority of DNA vaccinated patients displayed complete regression of their cervical lesions, as well as viral clearance, following DNA delivery. Other mechanical delivery approaches use physical force such as particle bombardment (gene gun) to deliver the DNA plasmids into targeted tissues or cells, with some clinical successes.[21-23] Delivery of a Hepatitis B DNA vaccine by particle bombardment resulted in sustained antibody titers in subjects who had previously failed to respond to a licensed subunit vaccine.[23] Needle-free pneumatic or jet injectors have also shown promise in both animal and human

clinical trials [24-27], and function by injecting a high-pressure, narrow stream of injection liquid into the epidermis or muscles of test subjects. In addition to these improved mechanical delivery methods, several other approaches are being explored to increase the immunogenicity of DNA vaccines in humans. Here we review three of these approaches which show promise for advancing DNA vaccines: non-mechanical delivery, inclusion of molecular adjuvants, and improvements in DNA vaccine vectors.

Non-Mechanical DNA Vaccine Delivery

As already mentioned, the greatest impediment to DNA vaccination is low immunogenicity due to difficulties in delivering DNA plasmid into the host cell. The transportation of DNA vaccine plasmids into cellular nuclei requires the crossing of several barriers. Vaccine plasmid must cross the phospholipid cellular membrane through endocytosis or pinocytosis, escape degradation in endosomes and lysosomes, survive cytosolic nucleases, and translocate across the nuclear envelope. In contrast to physical delivery systems, chemical delivery approaches use biopharmaceuticals to increase DNA vaccine transfection efficiency.

The use of liposomes as a carrier molecule has become a popular DNA vaccine delivery method as liposomes not only enhance transfection efficiency, but also have an adjuvant effect. Liposomes are spherical vesicles composed of phospholipids and cholesterol arranged into a lipid bilayer, allowing for fusion with cellular lipid membranes.[28] DNA plasmid can be either bound to the liposome surface, or encased within the hydrophobic core of the liposome. This facilitates delivery of the DNA vaccine plasmid into the cells. Importantly, lipid vesicles can be formulated as either unilamellar or multilamellar. Multilamellar vesicles allow for sustained delivery of vaccine over an extended period of time. While the use of liposomes for IM injection has resulted in some reactogenicity issues [29, 30], liposome/DNA vaccine complexes have

demonstrated an immunological benefit. IM injection of a liposome/influenza nucleoprotein formulation increased antibody titers 20-fold compared to vaccine alone.[31, 32] Boosting of antibody titers did not diminish the cytotoxic T cell response. Likewise, inclusion of a liposome formulation in a *P. falciparum* vaccine enhanced the IFN-γ production.[33, 34] An ensuing human trial involving DNA plasmids encoding the influenza H5 HA, nucleoprotein, and M2 genes reported cellular immune response rates and antibody titers comparable to that of the currently available inactivated protein-based H5 vaccines.[35] Additionally, liposomes have shown promise as a candidate for delivery of DNA vaccines to mucosal tissue.[36] A recent study demonstrated that vaccination with liposome encapsulated influenza A virus M1 induced both humoral and cellular immune responses that protected against respiratory infection.[36] Liposomes have also been shown to be an effective delivery method for intranasal DNA vaccination, conferring protective immune responses against infection.[37, 38]

DNA vaccine delivery can also be accomplished through the use of biodegradable polymeric micro- and nanoparticles consisting of amphiphilic molecules between 0.5-10 micrometers in size. Similar to loading of DNA plasmid on liposomes, plasmid molecules can be either encapsulated or adsorbed onto the surface of the nanoparticles.[39-42] These particles function as a carrier system, protecting the vaccine plasmid from degradation by extracellular deoxyribonucleases. In addition to shielding plasmid DNA from nucleases, micro- and nanoparticles promote the sustained release of vaccine instead of the bolus type of delivery characteristic of larger submicrometer complexes.[39, 43] High molecular weight cationic polymers have proven significantly more effective than cationic liposomes in aggregating DNA vaccine plasmid. Plasmid DNA immobilized within biodegradable chitosan-coated polymeric microspheres (ranging from 20 to 500 μm) can induce both mucosal and systemic immune

responses.[44] Microspheres may be delivered either by the oral or intraperitoneal route, allowing for direct transfection of dendritic cells (DC), thereby increasing DC activation. The benefits of microsphere formulations have been shown in mice, non-human primates, and humans [45-49] against a wide range of diseases including hepatitis B [50], tuberculosis [51], and cancer.[52] These results suggest that microparticle-based delivery systems are capable of significantly improving DNA vaccine immunogenicity, and boosting cellular and humoral immune responses.

The use of liposomes or nanoparticles appears to be safe and well tolerated in clinical studies. Microparticle-based delivery systems can increase gene expression, as well as, DNA vaccine immunogenicity. Although many of the earliest carrier formulations did not show a significant clinical benefit, more recent studies highlighted herein yielded promising clinical data. As microparticles can be prepared with significant structural diversity (size, surface charge, lipid content), they offer considerable flexibility of vaccine formulation. This allows for optimization of the vaccine based on the specific needs of the clinician.

Molecular Adjuvants

Another approach that has been effective in increasing DNA vaccine immunogenicity is the use of “vaccine cocktails” containing the DNA vaccine as well as plasmids encoding adjuvanting immunomodulatory proteins. Plasmid DNA contains unmethylated deoxycytidylate-phosphate-deoxyguanylate (CpG) motifs that function as a “built in” adjuvant.[53-59] Molecular adjuvant plasmids expressing cytokines, chemokines, or co-stimulatory molecules may be co-administered with the antigenic DNA vaccine plasmid. Cells transfected by molecular adjuvant plasmids secrete the adjuvant into the surrounding region, stimulating both local antigen presenting cells (APC) and cells in the draining lymph node. This results in durable, but low level, production of immune modulating cytokines that can tailor the immune response towards a

more desirable outcome without the concerns of a systemic cytokine storm. While human data is limited, a wide range of inflammatory and helper T cell cytokines have been studied, in conjunction with DNA vaccination, in small animal models.[60, 61] In particular, we have highlighted a few of the most prominent molecular adjuvants with demonstrated ability to increase DNA vaccine immunogenicity.[62] A more comprehensive list of molecular adjuvants is included in **Table 1**.

Plasmid-encoded cytokines

Cytokines are a class of immunoregulatory proteins that affect the behavior of other cells, and are critical for immune cell signaling. Cytokine-encoding genes can be delivered either as a separate plasmid, or as additional genes encoded within the antigen containing plasmid. The most extensively studied molecular adjuvant is Interleukin-2 (IL-2). IL-2 plays an essential role in the immune response by promoting the differentiation of naïve T cells into effector T cells, as well as driving the generation of memory T cell pools. It is also required for the proliferation of Natural Killer (NK) cells. Inclusion of IL-2 has resulted in improved immunogenicity for HIV [63-65], influenza [66], and SARS-CoV [67] anti-viral DNA vaccines. Interestingly, a therapeutic vaccine encoding for the BCR/ABL-pIRES genes of myeloid leukemia and IL-2 also demonstrated enhanced immune responses, suggesting that IL-2 molecular adjuvants have the capability of alleviating the symptoms of chronic infection.[68]

Similar to IL-2, IL-15 is a cytokine that induces NK and T cell proliferation. IL-15 is necessary for the generation of primary antigen-specific $CD4^+$ and $CD8^+$ T cell responses. It also plays a substantial role in establishment of memory $CD8^+$ T cell populations.[69-73] Results of small animal studies suggest that the adjuvant effect of IL-15 is most potent when delivered in tandem with other cytokines. For example, a synergistic effect was seen when IL-15 and IL-21 were co-

delivered with a DNA vaccine against *Toxoplasma gondii* infection.[74, 75] Additionally, sequential administration of IL-6, IL-7, and IL-15 genes augmented long-term $CD4^+$ T cell memory responses to a foot and mouth disease DNA vaccine.[76] Therefore, depending on the antigen, it may be necessary to deliver IL-15 in combination with other molecular adjuvants. Notably, a study in rhesus macaques suggests that delivery of an IL-15 encoding DNA vaccine itself resulted in increased proliferation of NK and T cells, with no adverse effects.[77] Another recent study demonstrated that co-vaccination of rhesus macaques with SIV pol plasmid and HIV env plasmid plus IL-15 allowed for faster control of viremia than the group not formulated with IL-15.[78] Moreover, macaques vaccinated with IL-15 exhibited increased T cell proliferation compared to those receiving the antigen plasmid alone, suggesting that IL-15 has a robust effect on T cell memory responses.

IL-12 is another pro-inflammatory cytokine secreted by both dendritic cells and monocytes. IL-12 plays an integral role in shaping the innate and adaptive immune responses to infection.[79-83] IL-12 signaling supports the secondary expansion of activated T helper 1 (T_{h1}) cells [79, 82, 84-86], resulting in high levels of antigen-specific $CD8^+$ T cells, and the expression of cytotoxic mediators such as interferon-γ (IFN-γ), granzyme B, and perforin.[82, 83] IL-12 was the first cytokine to be evaluated for use as a molecular adjuvant, and several studies have shown that inclusion of IL-12 expression plasmids within the vaccine formulation enhances T_{h1} immune responses.[87-95] Vaccination of mice with a bicistronic plasmid expressing IL-12 and *Yersinia pestis* resulted in increased mucosal IgA and serum IgG, providing significantly higher levels of protection against challenge than antigen-only groups.[96] Studies in rhesus macaques have shown similar increases in DNA vaccine immunogenicity. Co-vaccination with SIV gag and IL-12 allowed for dose sparing [97], as well as increased breadth of T cell responses.[89, 91, 98, 99]

Additionally, multiple human clinical studies utilizing vaccines adjuvanted with IL-12 have proven safe [100] and highly immunogenic, yielding high level $CD4^+$ and $CD8^+$ T cell responses.[87, 101, 102] Furthermore, inclusion of IL-12 expression plasmids can improve weakly immunogenic vaccines. A recent clinical study demonstrated that addition of IL-12 improved the immunogenicity of a Hepatitis B DNA vaccine, resulting in increased vaccine immunogenicity, as well as sustained memory T cell responses.[103]

The final immunomodulatory cytokine that has received considerable focus as a molecular adjuvant is granulocyte-macrophage colony stimulating factor (GM-CSF). GM-CSF recruits antigen presenting cells to the vaccination site and promotes DC maturation.[104] It has been successfully used in multiple DNA vaccines.[105-107] Plasmid-encoded GM-CSF, when co-delivered with a rabies virus DNA vaccine in mice, resulted in increased $CD4^+$ T cell responses, antibody production, and protection from lethal viral challenge.[108] Likewise, a bicistronic DNA vaccine encoding HIV-1 gp120 and GM-CSF recruited inflammatory cellular infiltrates and elicited a potent $CD4^+$ T cell response.[109] However, the benefit of GM-CSF molecular adjuvants remains unclear. Recent studies have shown that co-administration of GM-CSF plasmid with an antigen-encoding DNA vaccine can have deleterious effects. Co-delivery of GM-CSF suppressed the response to a DNA vaccine encoding Dengue virus type 1 and type 2, and also failed to improve the response elicited by a Hepatitis C vaccine.[110] Furthermore, inclusion of plasmid GM-CSF provided minimal adjuvant effect when co-administered with a malaria DNA vaccine in rhesus macaques.[111] Likewise, GM-CSF had no clear effect on T cell responses in patients receiving a melanoma DNA vaccine.[112] One possible explanation for these results is that high levels of GM-CSF can expand myeloid suppressor cell populations, and suppress the generation of adaptive immune responses. Alternatively, the lack of improved immunogenicity seen in

clinical trials may be due to the relative lack of GM-CSF receptors on rhesus and human APC compared to murine cells.[113] While no specific adverse effects have been reported, the use of GM-CSF as an adjuvant may require some fine-tuning, particularly if GM-CSF expression levels must be considered with regards to immunosuppression.

In addition to cytokine-encoding plasmids, several other methods for increasing DNA vaccine immunogenicity exist. The increased understanding of immune signaling pathways has led to the development of adjuvant plasmids encoding adhesion molecules, chemokines, costimulatory molecules, and Toll-like receptor (TLR) ligands. These molecular adjuvants have had some success in small animal models. For example, the innate immune signaling molecule TRIF increased the antibody response generated by a swine fever virus DNA vaccine.[114] Moreover, TRIF increased the protective activity of an influenza HA-encoding DNA vaccine.[115] Similar results were seen in studies encoding the dsRNA receptors MDA5 and RIG-I.[116, 117] Additionally, antigen-fusion constructs, whereby the antigen of interest is linked to a "carrier protein", can increase the immune visibility of the vaccine, and enhance DNA vaccine potency.[118-120]

A major advantage of DNA vaccination is the ability of multiple molecules such as molecular adjuvants to be inserted into the plasmid. Unlike the addition of recombinant cytokines, co-stimulatory molecules, and TLR ligands, which have a limited duration due to the short half-life of recombinant protein *in vivo*, molecular adjuvant-encoding plasmids will express protein for the same duration as the antigen, stimulating the immune system for a greater length of time. This can be done without fear of eliciting a cytokine storm, as generation of the adjuvanting signal will be localized to the site of vaccination. Of note, homologous recombination between plasmid-encoded cytokines and the host gene sequence does not appear to be a significant

concern, as multiple studies have shown that only extrachromosomal plasmid DNA has been identified following intramuscular injection.[121, 122] Furthermore, many current plasmids have been-codon optimized to improve gene expression in mammalian cells. This has resulted in changes to the cytokine gene sequence, limiting the possibility for homologous recombination and/or integration. Molecular adjuvants therefore show great promise for both increasing immunogenicity and extending the longevity of the immune response.

Improvements in DNA plasmid design

Plasmid DNA vectors contain functional elements, such as the origin of replication and selection markers, that are only required during the prokaryotic growth process in *E. coli*. These "bacterial region" elements (**Figure 2**) are no longer needed once cell culture is halted, and may have a negative effect on vaccine stability, uptake, and efficacy. Additionally, these elements can pose safety concerns, particularly if widely used antibiotic resistance markers are horizontally transmitted to host enteric bacteria populations.[123, 124]

These concerns have been addressed by development of small bacterial RNA-based antibiotic free selection markers.[124, 125] Noncoding RNA markers are preferable to protein markers since proteins, like antibiotic resistance markers, can be expressed in the host organism after vector transfection, or horizontally transmitted to host bacteria. Noncoding RNA markers are also very small (<200 basepairs) which decreases the overall vector size; this is advantageous since vector transfection efficiency is inversely related to vector size [126-128], perhaps because smaller vectors are more resistant to delivery associated shear forces [129] and may have improved nuclear localization since they are more motile in the cytoplasm.[130] Additionally, some bacterial region protein marker genes have been shown to dramatically reduce vector expression. For example, the TN5 derived NPT-II kanamycin resistance marker (kanR) gene in the pVAX1 vector

bacterial region significantly reduces transgene expression. Three groups have demonstrated that pVAX1 bacterial region mediated repression of transgene expression can be alleviated by replacement of the kanR gene with either a tRNA RNA selection marker, the RNA-OUT antisense RNA selection marker, or the endogenous pUC origin RNAI antisense RNA selection marker.[131-133] Consistent with this, removal of the pVAX1 bacterial region in a minicircle vector improved humoral and cellular immune responses up to 3 fold compared to a pVAX1 vector control.[134]

DNA vaccine vectors with dramatically higher transgene expression have recently been developed through identification of novel bacterial region and eukaryotic region vector configurations. Pioneering work by Mark Kay's laboratory at Stanford University demonstrated that bacterial regions larger than 1 kilobase silenced transgene expression in quiescent tissue such as the liver, likely due to untranscribed bacterial region mediated heterochromatin formation that spreads to the eukaryotic region and inactivates the promoter.[135-137] Minicircle vectors, in which the bacterial region is removed by the action of a phage recombinase during production, alleviated this silencing.[135, 136, 138] However, production of minicircle vectors is low yield and poorly scalable due to the required *in vivo* or *in vitro* recombination during manufacture.[139] In an effort to create alternative short bacterial region vectors that could be efficiently manufactured, the Mini-Intronic Plasmid (MIP) and Nanoplasmid™ vector plasmid platforms were developed. MIP vectors incorporate a RNA-OUT selection marker-pUC origin bacterial region within a 5' UTR intron. In this configuration the bacterial region is within the transcription unit and the downstream polyA signal is linked to the eukaryotic promoter without an intervening selection marker or replication origin. Nanoplasmid™ vectors are RNA-OUT selection marker vectors in which the large pUC bacterial replication origin is replaced by a

small R6K bacterial replication origin. In this configuration, the <500 basepair (bp) bacterial region separates the polyA signal and the eukaryotic promoter. Unlike minicircles, both MIP and Nanoplasmid™ RNA-OUT selection vectors can be efficiently manufactured in gram/liter yields without antibiotic selection.[140]

As expected, both vector platforms alleviate gene silencing in quiescent tissues similarly to minicircle vectors.[141, 142] However, unexpectedly both MIP and Nanoplasmid™ vectors dramatically improve overall gene expression up to 10 fold compared to plasmid and minicircle vectors in quiescent (liver) and non-quiescent tissues.[141, 142] The improved expression level after ID and IM delivery has application to improve DNA vaccination since increased expression level is correlative with improved humoral and cellular immune response.[62]

Another approach to improve DNA vaccines is to engineer the vector to increase innate immune activation. DNA vaccines are potent triggers of innate immunity. Various studies have determined several innate immune pathways are activated by DNA vaccination (**Figure 2**). Most of the intrinsic adjuvant effect of DNA is mediated by cytoplasmic innate immune receptors that nonspecifically recognize B DNA and activate Sting or Inflammasome mediated signaling [53, 143], but unmethylated CpG sequences specific for TLR9 activation may also be important for priming CD8 T cell responses.[144, 145] Along these lines, DNA vaccine vectors may be sequence modified to introduce immunostimulatory xxCGxx TLR9 agonists into the vector to increase innate immune activation. This approach has been used to improve DNA vaccine immunogenicity [58, 59, 146], but the results are variable. Some of the variability may be due to unintended inhibition of the eukaryotic promoter expression resulting from integration of CpG motifs into non-permissive sites in the vector.[125] As well, certain DNA delivery methods may not transfer DNA to the endosome as effectively as other deliveries (*e.g.* liposomes), preventing

unmethylated CpG interaction with, and activation of, TLR9. Part of the complexity is that optimal TLR9 activating xxCGxx motifs are species-specific; different xxCGxx agonist motifs differentially modulate the immune response [147] and many xxCGxx motifs are immunosuppressive.

An alternative strategy is to encode immunostimulatory RNA within the plasmid to increase innate immune activation. This approach has the potential advantage that additional innate immune pathways not normally stimulated by DNA alone are activated, resulting in polyvalent activation of multiple innate immune pathways to enhance immune activation.[148, 149] Like TLR9 for DNA, several innate immune TLRs for RNA are endosomal.[150] Activation of these receptors requires motif introduction into an expressed RNA, as well as cytoplasmic RNA shuttling into the endosome by autophagy. For example, 3'UTR incorporation of a 20 bp immunostimulatory ssRNA encoding D type CpG upstream of a 28 bp hairpin dsRNA resulted in a 4 fold increase in antigen reactive IgG titers [151], and a 2 fold increase in IFN-γ secreting $CD4^+$ and $CD8^+$ T cells.[152] Moreover, several RNA-sensing innate immune receptors such as RIG-I, MDA5 and DDX3 are cytoplasmic.[143] DNA vaccine expressed RNA can be used to target these receptors directly, without autophagy. Of these, RIG-I is of particular interest since RIG-I agonists have demonstrated adjuvant properties to improve the humoral response [153], humoral and $CD4^+$ T cell response [154, 155], and $CD8^+$ T cell response [153] to co-administered antigens.[156] In addition, RIG-I is ubiquitiously expressed in most tissues (expression of TLRs typically is restricted to immune cell subtypes) and certain RIG-I agonists that can be expressed in DNA vaccines (*e.g.* a blunt dsRNA with a 5' triphosphate) are structurally conserved between humans and mice. A DNA vaccine vector that co-expresses with antigen a RIG-I dsRNA agonist in a vector backbone

encoded RNA Polymerase III transcription unit (**Figure 2**) enhanced the humoral and $CD8^+$ T cell response after DNA vaccination.[117]

DNA vaccines encoding immunostimulatory sequences that selectively improve CTL responses to encoded antigen may have niche application in vaccines for intracellular pathogens or cancer. Innovations that increase transgene expression may be used to improve the performance of immunomodulatory molecular adjuvant plasmids, in addition to traditional antigen expressing DNA vaccine plasmids. Collectively, vector design innovations that improve transgene expression level and innate immune activation are complementary to improved mechanical and non-mechanical DNA vaccine delivery platforms. Combining improved vectors with liposome or polymeric particle non-mechanical delivery, or with needle free injector device delivery, has the potential to increase immunogenicity with these well tolerated, safe, delivery platforms.

Conclusion

While DNA vaccination provides several advantages over more conventional vaccination strategies, further optimization is necessary before it becomes the predominant strategy in human patients. Despite initial setbacks, significant progress has been made in overcoming the problem of low immunogenicity in humans. A clearer understanding of the immune mechanisms governing DNA vaccine immunogenicity has illuminated several pathways that may be useful in further improving DNA vaccine efficacy. A large catalogue of cytokines, chemokines, adhesion molecules, and transcription factors are in the process of being tested as molecular adjuvants, although it is likely that each will need to be carefully assessed for safety and tolerability. Likewise, continued development of vaccine delivery methods appears promising. New formulations exploiting sustained vaccine delivery methods, such as slow-releasing micropatches or multilamellar vesicles, are on the horizon. The strong appeal of needle-free injection and

353 mucosal delivery, the ease of design, and the recent clinical successes with DNA vaccines
354 suggests that this approach is on the precipice of redefining the field of vaccinology.

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References

1. Galvin TA, Muller J and Khan AS (2000) Effect of different promoters on immune responses elicited by HIV-1 gag/env multigenic DNA vaccine in Macaca mulatta and Macaca nemestrina. Vaccine 18:2566-83.
2. Huang MT and Gorman CM (1990) Intervening sequences increase efficiency of RNA 3' processing and accumulation of cytoplasmic RNA. Nucleic Acids Res 18:937-47.
3. Li L and Petrovsky N (2016) Molecular mechanisms for enhanced DNA vaccine immunogenicity. Expert Rev Vaccines 15:313-29. doi: 10.1586/14760584.2016.1124762
4. Goulder PJ, Rowland-Jones SL, McMichael AJ and Walker BD (1999) Anti-HIV cellular immunity: recent advances towards vaccine design. AIDS 13 Suppl A:S121-36.
5. Giri M, Ugen KE and Weiner DB (2004) DNA vaccines against human immunodeficiency virus type 1 in the past decade. Clin Microbiol Rev 17:370-89.
6. Wang R, Epstein J, Baraceros FM, Gorak EJ, Charoenvit Y, Carucci DJ, Hedstrom RC, Rahardjo N, Gay T, Hobart P, Stout R, Jones TR, Richie TL, Parker SE, Doolan DL, Norman J and Hoffman SL (2001) Induction of CD4(+) T cell-dependent CD8(+) type 1 responses in humans by a malaria DNA vaccine. Proc Natl Acad Sci U S A 98:10817-22. doi: 10.1073/pnas.181123498
7. Hollister K, Chen Y, Wang S, Wu H, Mondal A, Clegg N, Lu S and Dent A (2014) The role of follicular helper T cells and the germinal center in HIV-1 gp120 DNA prime and gp120 protein boost vaccination. Hum Vaccin Immunother 10:1985-92. doi: 10.4161/hv.28659
8. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A and Felgner PL (1990) Direct gene transfer into mouse muscle in vivo. Science 247:1465-8.
9. Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dwarki VJ, Gromkowski SH, Deck RR, DeWitt CM, Friedman A and et al. (1993) Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 259:1745-9.
10. Wang S, Kennedy JS, West K, Montefiori DC, Coley S, Lawrence J, Shen S, Green S, Rothman AL, Ennis FA, Arthos J, Pal R, Markham P and Lu S (2008) Cross-subtype antibody and cellular immune responses induced by a polyvalent DNA prime-protein boost HIV-1 vaccine in healthy human volunteers. Vaccine 26:3947-57.
11. Donnelly JJ, Martinez D, Jansen KU, Ellis RW, Montgomery DL and Liu MA (1996) Protection against papillomavirus with a polynucleotide vaccine. J Infect Dis 173:314-20.
12. Fuller DH and Haynes JR (1994) A qualitative progression in HIV type 1 glycoprotein 120-specific cytotoxic cellular and humoral immune responses in mice receiving a DNA-based glycoprotein 120 vaccine. AIDS Res Hum Retroviruses 10:1433-41. doi: 10.1089/aid.1994.10.1433
13. Dupuy LC, Richards MJ, Ellefsen B, Chau L, Luxembourg A, Hannaman D, Livingston BD and Schmaljohn CS (2011) A DNA vaccine for venezuelan equine encephalitis virus delivered by intramuscular electroporation elicits high levels of neutralizing antibodies in multiple animal models and provides protective immunity to mice and nonhuman primates. Clin Vaccine Immunol 18:707-16. doi: 10.1128/CVI.00030-11
14. Dupuy LC, Richards MJ, Reed DS and Schmaljohn CS (2010) Immunogenicity and protective efficacy of a DNA vaccine against Venezuelan equine encephalitis virus aerosol challenge in nonhuman primates. Vaccine 28:7345-50. doi: 10.1016/j.vaccine.2010.09.005
15. Grant-Klein RJ, Altamura LA, Badger CV, Bounds CE, Van Deusen NM, Kwilas SA, Vu HA, Warfield KL, Hooper JW, Hannaman D, Dupuy LC and Schmaljohn CS (2015) Codon-optimized filovirus DNA vaccines delivered by intramuscular electroporation protect cynomolgus macaques from lethal Ebola and Marburg virus challenges. Human Vaccines & Immunotherapeutics 11:1991-2004. doi: 10.1080/21645515.2015.1039757

16. Grant-Klein RJ, Van Deusen NM, Badger CV, Hannaman D, Dupuy LC and Schmaljohn CS (2012) A multiagent filovirus DNA vaccine delivered by intramuscular electroporation completely protects mice from ebola and Marburg virus challenge. Hum Vaccin Immunother 8:1703-6. doi: 10.4161/hv.21873
17. Bagarazzi ML, Yan J, Morrow MP, Shen X, Parker RL, Lee JC, Giffear M, Pankhong P, Khan AS, Broderick KE, Knott C, Lin F, Boyer JD, Draghia-Akli R, White CJ, Kim JJ, Weiner DB and Sardesai NY (2012) Immunotherapy against HPV16/18 generates potent TH1 and cytotoxic cellular immune responses. Sci Transl Med 4:155ra138. doi: 10.1126/scitranslmed.3004414
18. Vasan S, Hurley A, Schlesinger SJ, Hannaman D, Gardiner DF, Dugin DP, Boente-Carrera M, Vittorino R, Caskey M, Andersen J, Huang Y, Cox JH, Tarragona-Fiol T, Gill DK, Cheeseman H, Clark L, Dally L, Smith C, Schmidt C, Park HH, Kopycinski JT, Gilmour J, Fast P, Bernard R and Ho DD (2011) In vivo electroporation enhances the immunogenicity of an HIV-1 DNA vaccine candidate in healthy volunteers. PLoS One 6:e19252. doi: 10.1371/journal.pone.0019252
19. Low L, Mander A, McCann K, Dearnaley D, Tjelle T, Mathiesen I, Stevenson F and Ottensmeier CH (2009) DNA vaccination with electroporation induces increased antibody responses in patients with prostate cancer. Hum Gene Ther 20:1269-78. doi: 10.1089/hum.2009.067
20. Kim TJ, Jin HT, Hur SY, Yang HG, Seo YB, Hong SR, Lee CW, Kim S, Woo JW, Park KS, Hwang YY, Park J, Lee IH, Lim KT, Lee KH, Jeong MS, Surh CD, Suh YS, Park JS and Sung YC (2014) Clearance of persistent HPV infection and cervical lesion by therapeutic DNA vaccine in CIN3 patients. Nat Commun 5:5317. doi: 10.1038/ncomms6317
21. Tang DC, DeVit M and Johnston SA (1992) Genetic immunization is a simple method for eliciting an immune response. Nature 356:152-4. doi: 10.1038/356152a0
22. Roy MJ, Wu MS, Barr LJ, Fuller JT, Tussey LG, Speller S, Culp J, Burkholder JK, Swain WF, Dixon RM, Widera G, Vessey R, King A, Ogg G, Gallimore A, Haynes JR and Heydenburg Fuller D (2000) Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. Vaccine 19:764-78.
23. Rottinghaus ST, Poland GA, Jacobson RM, Barr LJ and Roy MJ (2003) Hepatitis B DNA vaccine induces protective antibody responses in human non-responders to conventional vaccination. Vaccine 21:4604-8.
24. Choi AH, Smiley K, Basu M, McNeal MM, Shao M, Bean JA, Clements JD, Stout RR and Ward RL (2007) Protection of mice against rotavirus challenge following intradermal DNA immunization by Biojector needle-free injection. Vaccine 25:3215-8. doi: 10.1016/j.vaccine.2007.01.035
25. Ledgerwood JE, Hu Z, Gordon IJ, Yamshchikov G, Enama ME, Plummer S, Bailer R, Pearce MB, Tumpey TM, Koup RA, Mascola JR, Nabel GJ, Graham BS, Vrc and Teams VRCS (2012) Influenza virus h5 DNA vaccination is immunogenic by intramuscular and intradermal routes in humans. Clin Vaccine Immunol 19:1792-7. doi: 10.1128/CVI.05663-11
26. Aguiar JC, Hedstrom RC, Rogers WO, Charoenvit Y, Sacci JB, Jr., Lanar DE, Majam VF, Stout RR and Hoffman SL (2001) Enhancement of the immune response in rabbits to a malaria DNA vaccine by immunization with a needle-free jet device. Vaccine 20:275-80.
27. Trimble C, Lin CT, Hung CF, Pai S, Juang J, He L, Gillison M, Pardoll D, Wu L and Wu TC (2003) Comparison of the CD8+ T cell responses and antitumor effects generated by DNA vaccine administered through gene gun, biojector, and syringe. Vaccine 21:4036-42.
28. Karkada M, Weir GM, Quinton T, Fuentes-Ortega A and Mansour M (2010) A liposome-based platform, VacciMax, and its modified water-free platform DepoVax enhance efficacy of in vivo nucleic acid delivery. Vaccine 28:6176-82. doi: 10.1016/j.vaccine.2010.07.025
29. Fries LF, Gordon DM, Richards RL, Egan JE, Hollingdale MR, Gross M, Silverman C and Alving CR (1992) Liposomal malaria vaccine in humans: a safe and potent adjuvant strategy. Proc Natl Acad Sci U S A 89:358-62.

30. Zollinger WD, Babcock JG, Moran EE, Brandt BL, Matyas GR, Wassef NM and Alving CR (2012) Phase I study of a Neisseria meningitidis liposomal vaccine containing purified outer membrane proteins and detoxified lipooligosaccharide. Vaccine 30:712-21. doi: 10.1016/j.vaccine.2011.11.084
31. Hartikka J, Bozoukova V, Ferrari M, Sukhu L, Enas J, Sawdey M, Wloch MK, Tonsky K, Norman J, Manthorpe M and Wheeler CJ (2001) Vaxfectin enhances the humoral immune response to plasmid DNA-encoded antigens. Vaccine 19:1911-23.
32. Reyes L, Hartikka J, Bozoukova V, Sukhu L, Nishioka W, Singh G, Ferrari M, Enas J, Wheeler CJ, Manthorpe M and Wloch MK (2001) Vaxfectin enhances antigen specific antibody titers and maintains Th1 type immune responses to plasmid DNA immunization. Vaccine 19:3778-86.
33. Sedegah M, Rogers WO, Belmonte A, Belmonte M, Banania G, Patterson N, Ferrari M, Kaslow DC, Carucci DJ, Richie TL and Doolan DL (2006) Vaxfectin enhances immunogenicity and protective efficacy of P. yoelii circumsporozoite DNA vaccines. Vaccine 24:1921-7. doi: 10.1016/j.vaccine.2005.10.041
34. Sedegah M, Rogers WO, Belmonte M, Belmonte A, Banania G, Patterson NB, Rusalov D, Ferrari M, Richie TL and Doolan DL (2010) Vaxfectin enhances both antibody and in vitro T cell responses to each component of a 5-gene Plasmodium falciparum plasmid DNA vaccine mixture administered at low doses. Vaccine 28:3055-65. doi: 10.1016/j.vaccine.2009.10.044
35. Smith LR, Wloch MK, Ye M, Reyes LR, Boutsaboualoy S, Dunne CE, Chaplin JA, Rusalov D, Rolland AP, Fisher CL, Al-Ibrahim MS, Kabongo ML, Steigbigel R, Belshe RB, Kitt ER, Chu AH and Moss RB (2010) Phase 1 clinical trials of the safety and immunogenicity of adjuvanted plasmid DNA vaccines encoding influenza A virus H5 hemagglutinin. Vaccine 28:2565-72. doi: 10.1016/j.vaccine.2010.01.029
36. Liu J, Wu J, Wang B, Zeng S, Qi F, Lu C, Kimura Y and Liu B (2014) Oral vaccination with a liposome-encapsulated influenza DNA vaccine protects mice against respiratory challenge infection. J Med Virol 86:886-94. doi: 10.1002/jmv.23768
37. Ma J, Wang H, Zheng X, Xue X, Wang B, Wu H, Zhang K, Fan S, Wang T, Li N, Zhao Y, Gao Y, Yang S and Xia X (2014) CpG/Poly (I:C) mixed adjuvant priming enhances the immunogenicity of a DNA vaccine against eastern equine encephalitis virus in mice. Int Immunopharmacol 19:74-80. doi: 10.1016/j.intimp.2014.01.002
38. Chen L, Zhu J, Li Y, Lu J, Gao L, Xu H, Fan M and Yang X (2013) Enhanced nasal mucosal delivery and immunogenicity of anti-caries DNA vaccine through incorporation of anionic liposomes in chitosan/DNA complexes. PLoS One 8:e71953. doi: 10.1371/journal.pone.0071953
39. Basarkar A, Devineni D, Palaniappan R and Singh J (2007) Preparation, characterization, cytotoxicity and transfection efficiency of poly(DL-lactide-co-glycolide) and poly(DL-lactic acid) cationic nanoparticles for controlled delivery of plasmid DNA. Int J Pharm 343:247-54. doi: 10.1016/j.ijpharm.2007.05.023
40. Mok H and Park TG (2008) Direct plasmid DNA encapsulation within PLGA nanospheres by single oil-in-water emulsion method. Eur J Pharm Biopharm 68:105-11. doi: 10.1016/j.ejpb.2007.04.022
41. Hao T, McKeever U and Hedley ML (2000) Biological potency of microsphere encapsulated plasmid DNA. J Control Release 69:249-59.
42. Singh M, Briones M, Ott G and O'Hagan D (2000) Cationic microparticles: A potent delivery system for DNA vaccines. Proc Natl Acad Sci U S A 97:811-6.
43. Aral C and Akbuga J (2003) Preparation and in vitro transfection efficiency of chitosan microspheres containing plasmid DNA:poly(L-lysine) complexes. J Pharm Pharm Sci 6:321-6.
44. Alexakis T, Boadi DK, Quong D, Groboillot A, O'Neill I, Poncelet D and Neufeld RJ (1995) Microencapsulation of DNA within alginate microspheres and crosslinked chitosan membranes for in vivo application. Appl Biochem Biotechnol 50:93-106.

45. Herrmann JE, Chen SC, Jones DH, Tinsley-Bown A, Fynan EF, Greenberg HB and Farrar GH (1999) Immune responses and protection obtained by oral immunization with rotavirus VP4 and VP7 DNA vaccines encapsulated in microparticles. Virology 259:148-53. doi: 10.1006/viro.1999.9751
46. Kaur R, Rauthan M and Vrati S (2004) Immunogenicity in mice of a cationic microparticle-adsorbed plasmid DNA encoding Japanese encephalitis virus envelope protein. Vaccine 22:2776-82. doi: 10.1016/j.vaccine.2004.01.040
47. Otten GR, Schaefer M, Doe B, Liu H, Srivastava I, Megede J, Kazzaz J, Lian Y, Singh M, Ugozzoli M, Montefiori D, Lewis M, Driver DA, Dubensky T, Polo JM, Donnelly J, O'Hagan DT, Barnett S and Ulmer JB (2005) Enhanced potency of plasmid DNA microparticle human immunodeficiency virus vaccines in rhesus macaques by using a priming-boosting regimen with recombinant proteins. J Virol 79:8189-200. doi: 10.1128/JVI.79.13.8189-8200.2005
48. Wang F, He XW, Jiang L, Ren D, He Y, Li DA and Sun SH (2006) Enhanced immunogenicity of microencapsulated multiepitope DNA vaccine encoding T and B cell epitopes of foot-and-mouth disease virus in mice. Vaccine 24:2017-26. doi: 10.1016/j.vaccine.2005.11.042
49. Minigo G, Scholzen A, Tang CK, Hanley JC, Kalkanidis M, Pietersz GA, Apostolopoulos V and Plebanski M (2007) Poly-L-lysine-coated nanoparticles: a potent delivery system to enhance DNA vaccine efficacy. Vaccine 25:1316-27. doi: 10.1016/j.vaccine.2006.09.086
50. He X, Jiang L, Wang F, Xiao Z, Li J, Liu LS, Li D, Ren D, Jin X, Li K, He Y, Shi K, Guo Y, Zhang Y and Sun S (2005) Augmented humoral and cellular immune responses to hepatitis B DNA vaccine adsorbed onto cationic microparticles. J Control Release 107:357-72. doi: 10.1016/j.jconrel.2005.06.020
51. Mollenkopf HJ, Dietrich G, Fensterle J, Grode L, Diehl KD, Knapp B, Singh M, O'Hagan DT, Ulmer JB and Kaufmann SH (2004) Enhanced protective efficacy of a tuberculosis DNA vaccine by adsorption onto cationic PLG microparticles. Vaccine 22:2690-5.
52. Luo Y, O'Hagan D, Zhou H, Singh M, Ulmer J, Reisfeld RA, James Primus F and Xiang R (2003) Plasmid DNA encoding human carcinoembryonic antigen (CEA) adsorbed onto cationic microparticles induces protective immunity against colon cancer in CEA-transgenic mice. Vaccine 21:1938-47.
53. Ishii KJ, Kawagoe T, Koyama S, Matsui K, Kumar H, Kawai T, Uematsu S, Takeuchi O, Takeshita F, Coban C and Akira S (2008) TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. Nature 451:725-9. doi: 10.1038/nature06537
54. Okabe Y, Kawane K, Akira S, Taniguchi T and Nagata S (2005) Toll-like receptor-independent gene induction program activated by mammalian DNA escaped from apoptotic DNA degradation. J Exp Med 202:1333-9. doi: 10.1084/jem.20051654
55. Stetson DB and Medzhitov R (2006) Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. Immunity 24:93-103. doi: 10.1016/j.immuni.2005.12.003
56. Spies B, Hochrein H, Vabulas M, Huster K, Busch DH, Schmitz F, Heit A and Wagner H (2003) Vaccination with plasmid DNA activates dendritic cells via Toll-like receptor 9 (TLR9) but functions in TLR9-deficient mice. J Immunol 171:5908-12.
57. Babiuk S, Mookherjee N, Pontarollo R, Griebel P, van Drunen Littel-van den Hurk S, Hecker R and Babiuk L (2004) TLR9-/- and TLR9+/+ mice display similar immune responses to a DNA vaccine. Immunology 113:114-20. doi: 10.1111/j.1365-2567.2004.01938.x
58. Kojima Y, Xin KQ, Ooki T, Hamajima K, Oikawa T, Shinoda K, Ozaki T, Hoshino Y, Jounai N, Nakazawa M, Klinman D and Okuda K (2002) Adjuvant effect of multi-CpG motifs on an HIV-1 DNA vaccine. Vaccine 20:2857-65.
59. Coban C, Ishii KJ, Gursel M, Klinman DM and Kumar N (2005) Effect of plasmid backbone modification by different human CpG motifs on the immunogenicity of DNA vaccine vectors. J Leukoc Biol 78:647-55. doi: 10.1189/jlb.1104627
60. Abdulhaqq SA and Weiner DB (2008) DNA vaccines: developing new strategies to enhance immune responses. Immunol Res 42:219-32. doi: 10.1007/s12026-008-8076-3

61. Laddy DJ and Weiner DB (2006) From plasmids to protection: a review of DNA vaccines against infectious diseases. Int Rev Immunol 25:99-123. doi: 10.1080/08830180600785827
62. Flingai S, Czerwonko M, Goodman J, Kudchodkar SB, Muthumani K and Weiner DB (2013) Synthetic DNA vaccines: improved vaccine potency by electroporation and co-delivered genetic adjuvants. Front Immunol 4:354. doi: 10.3389/fimmu.2013.00354
63. Kim JJ, Nottingham LK, Wilson DM, Bagarazzi ML, Tsai A, Morrison LD, Javadian A, Chalian AA, Agadjanyan MG and Weiner DB (1998) Engineering DNA vaccines via co-delivery of co-stimulatory molecule genes. Vaccine 16:1828-35.
64. Kim JJ, Simbiri KA, Sin JI, Dang K, Oh J, Dentchev T, Lee D, Nottingham LK, Chalian AA, McCallus D, Ciccarelli R, Agadjanyan MG and Weiner DB (1999) Cytokine molecular adjuvants modulate immune responses induced by DNA vaccine constructs for HIV-1 and SIV. J Interferon Cytokine Res 19:77-84. doi: 10.1089/107999099314441
65. Barouch DH, Truitt DM and Letvin NL (2004) Expression kinetics of the interleukin-2/immunoglobulin (IL-2/Ig) plasmid cytokine adjuvant. Vaccine 22:3092-7. doi: 10.1016/j.vaccine.2004.01.065
66. Henke A, Rohland N, Zell R and Wutzler P (2006) Co-expression of interleukin-2 by a bicistronic plasmid increases the efficacy of DNA immunization to prevent influenza virus infections. Intervirology 49:249-52. doi: 10.1159/000092487
67. Hu H, Tao L, Wang Y, Chen L, Yang J and Wang H (2009) Enhancing immune responses against SARS-CoV nucleocapsid DNA vaccine by co-inoculating interleukin-2 expressing vector in mice. Biotechnol Lett 31:1685-93. doi: 10.1007/s10529-009-0061-y
68. Qin Y, Tian H, Wang G, Lin C and Li Y (2013) A BCR/ABL-hIL-2 DNA vaccine enhances the immune responses in BALB/c mice. Biomed Res Int 2013:136492. doi: 10.1155/2013/136492
69. McGill J, Van Rooijen N and Legge KL (2010) IL-15 trans-presentation by pulmonary dendritic cells promotes effector CD8 T cell survival during influenza virus infection. J Exp Med 207:521-34. doi: 10.1084/jem.20091711
70. Schluns KS, Williams K, Ma A, Zheng XX and Lefrancois L (2002) Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells. J Immunol 168:4827-31.
71. Yajima T, Yoshihara K, Nakazato K, Kumabe S, Koyasu S, Sad S, Shen H, Kuwano H and Yoshikai Y (2006) IL-15 regulates CD8+ T cell contraction during primary infection. J Immunol 176:507-15.
72. Combe CL, Moretto MM, Schwartzman JD, Gigley JP, Bzik DJ and Khan IA (2006) Lack of IL-15 results in the suboptimal priming of CD4+ T cell response against an intracellular parasite. Proc Natl Acad Sci U S A 103:6635-40. doi: 10.1073/pnas.0506180103
73. Ruckert R, Brandt K, Bulanova E, Mirghomizadeh F, Paus R and Bulfone-Paus S (2003) Dendritic cell-derived IL-15 controls the induction of CD8 T cell immune responses. Eur J Immunol 33:3493-503. doi: 10.1002/eji.200324545
74. Chen J, Li ZY, Huang SY, Petersen E, Song HQ, Zhou DH and Zhu XQ (2014) Protective efficacy of Toxoplasma gondii calcium-dependent protein kinase 1 (TgCDPK1) adjuvated with recombinant IL-15 and IL-21 against experimental toxoplasmosis in mice. BMC Infect Dis 14:487. doi: 10.1186/1471-2334-14-487
75. Li ZY, Chen J, Petersen E, Zhou DH, Huang SY, Song HQ and Zhu XQ (2014) Synergy of mIL-21 and mIL-15 in enhancing DNA vaccine efficacy against acute and chronic Toxoplasma gondii infection in mice. Vaccine 32:3058-65. doi: 10.1016/j.vaccine.2014.03.042
76. Su B, Wang J, Zhao G, Wang X, Li J and Wang B (2012) Sequential administration of cytokine genes to enhance cellular immune responses and CD4 (+) T memory cells during DNA vaccination. Hum Vaccin Immunother 8:1659-67. doi: 10.4161/hv.22105
77. Bergamaschi C, Kulkarni V, Rosati M, Alicea C, Jalah R, Chen S, Bear J, Sardesai NY, Valentin A, Felber BK and Pavlakis GN (2015) Intramuscular delivery of heterodimeric IL-15 DNA in macaques

602 produces systemic levels of bioactive cytokine inducing proliferation of NK and T cells. Gene Ther 22:76-
603 86. doi: 10.1038/gt.2014.84
604 78. Boyer JD, Robinson TM, Kutzler MA, Vansant G, Hokey DA, Kumar S, Parkinson R, Wu L, Sidhu
605 MK, Pavlakis GN, Felber BK, Brown C, Silvera P, Lewis MG, Monforte J, Waldmann TA, Eldridge J and
606 Weiner DB (2007) Protection against simian/human immunodeficiency virus (SHIV) 89.6P in macaques
607 after coimmunization with SHIV antigen and IL-15 plasmid. Proc Natl Acad Sci U S A 104:18648-53. doi:
608 10.1073/pnas.0709198104
609 79. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A and Murphy KM (1993) Development of
610 TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science 260:547-9.
611 80. Sypek JP, Chung CL, Mayor SE, Subramanyam JM, Goldman SJ, Sieburth DS, Wolf SF and Schaub
612 RG (1993) Resolution of cutaneous leishmaniasis: interleukin 12 initiates a protective T helper type 1
613 immune response. J Exp Med 177:1797-802.
614 81. Macatonia SE, Hosken NA, Litton M, Vieira P, Hsieh CS, Culpepper JA, Wysocka M, Trinchieri G,
615 Murphy KM and O'Garra A (1995) Dendritic cells produce IL-12 and direct the development of Th1 cells
616 from naive CD4+ T cells. J Immunol 154:5071-9.
617 82. Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, Loudon R, Sherman F, Perussia B and
618 Trinchieri G (1989) Identification and purification of natural killer cell stimulatory factor (NKSF), a
619 cytokine with multiple biologic effects on human lymphocytes. J Exp Med 170:827-45.
620 83. Trinchieri G (1998) Interleukin-12: a cytokine at the interface of inflammation and immunity.
621 Adv Immunol 70:83-243.
622 84. Hunter CA, Chizzonite R and Remington JS (1995) IL-1 beta is required for IL-12 to induce
623 production of IFN-gamma by NK cells. A role for IL-1 beta in the T cell-independent mechanism of
624 resistance against intracellular pathogens. J Immunol 155:4347-54.
625 85. Chan SH, Kobayashi M, Santoli D, Perussia B and Trinchieri G (1992) Mechanisms of IFN-gamma
626 induction by natural killer cell stimulatory factor (NKSF/IL-12). Role of transcription and mRNA stability
627 in the synergistic interaction between NKSF and IL-2. J Immunol 148:92-8.
628 86. Manetti R, Gerosa F, Giudizi MG, Biagiotti R, Parronchi P, Piccinni MP, Sampognaro S, Maggi E,
629 Romagnani S, Trinchieri G and et al. (1994) Interleukin 12 induces stable priming for interferon gamma
630 (IFN-gamma) production during differentiation of human T helper (Th) cells and transient IFN-gamma
631 production in established Th2 cell clones. J Exp Med 179:1273-83.
632 87. Kalams SA, Parker SD, Elizaga M, Metch B, Edupuganti S, Hural J, De Rosa S, Carter DK, Rybczyk
633 K, Frank I, Fuchs J, Koblin B, Kim DH, Joseph P, Keefer MC, Baden LR, Eldridge J, Boyer J, Sherwat A,
634 Cardinali M, Allen M, Pensiero M, Butler C, Khan AS, Yan J, Sardesai NY, Kublin JG, Weiner DB and
635 Network NHVT (2013) Safety and comparative immunogenicity of an HIV-1 DNA vaccine in combination
636 with plasmid interleukin 12 and impact of intramuscular electroporation for delivery. J Infect Dis
637 208:818-29. doi: 10.1093/infdis/jit236
638 88. Kim JJ, Ayyavoo V, Bagarazzi ML, Chattergoon M, Boyer JD, Wang B and Weiner DB (1997)
639 Development of a multicomponent candidate vaccine for HIV-1. Vaccine 15:879-83.
640 89. Hirao LA, Wu L, Khan AS, Hokey DA, Yan J, Dai A, Betts MR, Draghia-Akli R and Weiner DB (2008)
641 Combined effects of IL-12 and electroporation enhances the potency of DNA vaccination in macaques.
642 Vaccine 26:3112-20. doi: 10.1016/j.vaccine.2008.02.036
643 90. Boyer JD, Robinson TM, Kutzler MA, Parkinson R, Calarota SA, Sidhu MK, Muthumani K, Lewis M,
644 Pavlakis G, Felber B and Weiner D (2005) SIV DNA vaccine co-administered with IL-12 expression plasmid
645 enhances CD8 SIV cellular immune responses in cynomolgus macaques. J Med Primatol 34:262-70. doi:
646 10.1111/j.1600-0684.2005.00124.x
647 91. Chong SY, Egan MA, Kutzler MA, Megati S, Masood A, Roopchard V, Garcia-Hand D, Montefiori
648 DC, Quiroz J, Rosati M, Schadeck EB, Boyer JD, Pavlakis GN, Weiner DB, Sidhu M, Eldridge JH and Israel
649 ZR (2007) Comparative ability of plasmid IL-12 and IL-15 to enhance cellular and humoral immune

responses elicited by a SIVgag plasmid DNA vaccine and alter disease progression following SHIV(89.6P) challenge in rhesus macaques. Vaccine 25:4967-82. doi: 10.1016/j.vaccine.2006.11.070
92. Robinson TM, Sidhu MK, Pavlakis GN, Felber BK, Silvera P, Lewis MG, Eldridge J, Weiner DB and Boyer JD (2007) Macaques co-immunized with SIVgag/pol-HIVenv and IL-12 plasmid have increased cellular responses. J Med Primatol 36:276-84. doi: 10.1111/j.1600-0684.2007.00245.x
93. Bagley KC, Schwartz JA, Andersen H, Eldridge JH, Xu R, Ota-Setlik A, Geltz JJ, Halford WP and Fouts TR (2017) An Interleukin 12 Adjuvanted Herpes Simplex Virus 2 DNA Vaccine Is More Protective Than a Glycoprotein D Subunit Vaccine in a High-Dose Murine Challenge Model. Viral Immunol. doi: 10.1089/vim.2016.0136
94. Morrow MP, Yan J, Pankhong P, Ferraro B, Lewis MG, Khan AS, Sardesai NY and Weiner DB (2010) Unique Th1/Th2 phenotypes induced during priming and memory phases by use of interleukin-12 (IL-12) or IL-28B vaccine adjuvants in rhesus macaques. Clin Vaccine Immunol 17:1493-9. doi: 10.1128/CVI.00181-10
95. Bhaumik S, Basu R, Sen S, Naskar K and Roy S (2009) KMP-11 DNA immunization significantly protects against L. donovani infection but requires exogenous IL-12 as an adjuvant for comparable protection against L. major. Vaccine 27:1306-16. doi: 10.1016/j.vaccine.2008.12.053
96. Yamanaka H, Hoyt T, Yang X, Golden S, Bosio CM, Crist K, Becker T, Maddaloni M and Pascual DW (2008) A nasal interleukin-12 DNA vaccine coexpressing Yersinia pestis F1-V fusion protein confers protection against pneumonic plague. Infect Immun 76:4564-73. doi: 10.1128/IAI.00581-08
97. Schadeck EB, Sidhu M, Egan MA, Chong SY, Piacente P, Masood A, Garcia-Hand D, Cappello S, Roopchand V, Megati S, Quiroz J, Boyer JD, Felber BK, Pavlakis GN, Weiner DB, Eldridge JH and Israel ZR (2006) A dose sparing effect by plasmid encoded IL-12 adjuvant on a SIVgag-plasmid DNA vaccine in rhesus macaques. Vaccine 24:4677-87. doi: 10.1016/j.vaccine.2005.10.035
98. Halwani R, Boyer JD, Yassine-Diab B, Haddad EK, Robinson TM, Kumar S, Parkinson R, Wu L, Sidhu MK, Phillipson-Weiner R, Pavlakis GN, Felber BK, Lewis MG, Shen A, Siliciano RF, Weiner DB and Sekaly RP (2008) Therapeutic vaccination with simian immunodeficiency virus (SIV)-DNA + IL-12 or IL-15 induces distinct CD8 memory subsets in SIV-infected macaques. J Immunol 180:7969-79.
99. Li J, Valentin A, Kulkarni V, Rosati M, Beach RK, Alicea C, Hannaman D, Reed SG, Felber BK and Pavlakis GN (2013) HIV/SIV DNA vaccine combined with protein in a co-immunization protocol elicits highest humoral responses to envelope in mice and macaques. Vaccine 31:3747-55. doi: 10.1016/j.vaccine.2013.04.037
100. Cha E and Daud A (2012) Plasmid IL-12 electroporation in melanoma. Hum Vaccin Immunother 8:1734-8. doi: 10.4161/hv.22573
101. Kalams SA, Goulder PJ, Shea AK, Jones NG, Trocha AK, Ogg GS and Walker BD (1999) Levels of human immunodeficiency virus type 1-specific cytotoxic T-lymphocyte effector and memory responses decline after suppression of viremia with highly active antiretroviral therapy. J Virol 73:6721-8.
102. Kalams SA, Parker S, Jin X, Elizaga M, Metch B, Wang M, Hural J, Lubeck M, Eldridge J, Cardinali M, Blattner WA, Sobieszczyk M, Suriyanon V, Kalichman A, Weiner DB, Baden LR and Network NHVT (2012) Safety and immunogenicity of an HIV-1 gag DNA vaccine with or without IL-12 and/or IL-15 plasmid cytokine adjuvant in healthy, HIV-1 uninfected adults. PLoS One 7:e29231. doi: 10.1371/journal.pone.0029231
103. Yang SH, Lee CG, Park SH, Im SJ, Kim YM, Son JM, Wang JS, Yoon SK, Song MK, Ambrozaitis A, Kharchenko N, Yun YD, Kim CM, Kim CY, Lee SH, Kim BM, Kim WB and Sung YC (2006) Correlation of antiviral T-cell responses with suppression of viral rebound in chronic hepatitis B carriers: a proof-of-concept study. Gene Ther 13:1110-7. doi: 10.1038/sj.gt.3302751
104. Haddad D, Ramprakash J, Sedegah M, Charoenvit Y, Baumgartner R, Kumar S, Hoffman SL and Weiss WR (2000) Plasmid vaccine expressing granulocyte-macrophage colony-stimulating factor attracts infiltrates including immature dendritic cells into injected muscles. J Immunol 165:3772-81.

105. Weiss WR, Ishii KJ, Hedstrom RC, Sedegah M, Ichino M, Barnhart K, Klinman DM and Hoffman SL (1998) A plasmid encoding murine granulocyte-macrophage colony-stimulating factor increases protection conferred by a malaria DNA vaccine. J Immunol 161:2325-32.
106. Ahlers JD, Belyakov IM, Terabe M, Koka R, Donaldson DD, Thomas EK and Berzofsky JA (2002) A push-pull approach to maximize vaccine efficacy: abrogating suppression with an IL-13 inhibitor while augmenting help with granulocyte/macrophage colony-stimulating factor and CD40L. Proc Natl Acad Sci U S A 99:13020-5. doi: 10.1073/pnas.192251199
107. Yoon HA, Aleyas AG, George JA, Park SO, Han YW, Lee JH, Cho JG and Eo SK (2006) Cytokine GM-CSF genetic adjuvant facilitates prophylactic DNA vaccine against pseudorabies virus through enhanced immune responses. Microbiol Immunol 50:83-92.
108. Xiang Z and Ertl HC (1995) Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. Immunity 2:129-35.
109. Santana VC, Almeida RR, Ribeiro SP, Ferreira LC, Kalil J, Rosa DS and Cunha-Neto E (2015) Co-administration of plasmid-encoded granulocyte-macrophage colony-stimulating factor increases human immunodeficiency virus-1 DNA vaccine-induced polyfunctional CD4+ T-cell responses. Mem Inst Oswaldo Cruz 110:1010-6. doi: 10.1590/0074-02760150283
110. Chen H, Gao N, Wu J, Zheng X, Li J, Fan D and An J (2014) Variable effects of the co-administration of a GM-CSF-expressing plasmid on the immune response to flavivirus DNA vaccines in mice. Immunol Lett 162:140-8. doi: 10.1016/j.imlet.2014.08.005
111. Kumar S, Villinger F, Oakley M, Aguiar JC, Jones TR, Hedstrom RC, Gowda K, Chute J, Stowers A, Kaslow DC, Thomas EK, Tine J, Klinman D, Hoffman SL and Weiss WW (2002) A DNA vaccine encoding the 42 kDa C-terminus of merozoite surface protein 1 of Plasmodium falciparum induces antibody, interferon-gamma and cytotoxic T cell responses in rhesus monkeys: immuno-stimulatory effects of granulocyte macrophage-colony stimulating factor. Immunol Lett 81:13-24.
112. Cassaday RD, Sondel PM, King DM, Macklin MD, Gan J, Warner TF, Zuleger CL, Bridges AJ, Schalch HG, Kim KM, Hank JA, Mahvi DM and Albertini MR (2007) A phase I study of immunization using particle-mediated epidermal delivery of genes for gp100 and GM-CSF into uninvolved skin of melanoma patients. Clin Cancer Res 13:540-9. doi: 10.1158/1078-0432.CCR-06-2039
113. Barouch DH, Letvin NL and Seder RA (2004) The role of cytokine DNAs as vaccine adjuvants for optimizing cellular immune responses. Immunol Rev 202:266-74. doi: 10.1111/j.0105-2896.2004.00200.x
114. Wan C, Yi L, Yang Z, Yang J, Shao H, Zhang C and Pan Z (2010) The Toll-like receptor adaptor molecule TRIF enhances DNA vaccination against classical swine fever. Vet Immunol Immunopathol 137:47-53. doi: 10.1016/j.vetimm.2010.04.008
115. Takeshita F, Tanaka T, Matsuda T, Tozuka M, Kobiyama K, Saha S, Matsui K, Ishii KJ, Coban C, Akira S, Ishii N, Suzuki K, Klinman DM, Okuda K and Sasaki S (2006) Toll-like receptor adaptor molecules enhance DNA-raised adaptive immune responses against influenza and tumors through activation of innate immunity. J Virol 80:6218-24. doi: 10.1128/JVI.00121-06
116. Liniger M, Summerfield A and Ruggli N (2012) MDA5 can be exploited as efficacious genetic adjuvant for DNA vaccination against lethal H5N1 influenza virus infection in chickens. PLoS One 7:e49952. doi: 10.1371/journal.pone.0049952
117. Luke JM, Simon GG, Soderholm J, Errett JS, August JT, Gale M, Jr., Hodgson CP and Williams JA (2011) Coexpressed RIG-I agonist enhances humoral immune response to influenza virus DNA vaccine. J Virol 85:1370-83. doi: 10.1128/JVI.01250-10
118. Yang B, Yang A, Peng S, Pang X, Roden RB, Wu TC and Hung CF (2015) Co-administration with DNA encoding papillomavirus capsid proteins enhances the antitumor effects generated by therapeutic HPV DNA vaccination. Cell Biosci 5:35. doi: 10.1186/s13578-015-0025-y

119. Massa S, Paolini F, Spano L, Franconi R and Venuti A (2011) Mutants of plant genes for developing cancer vaccines. Hum Vaccin 7 Suppl:147-55.
120. Kim JW, Hung CF, Juang J, He L, Kim TW, Armstrong DK, Pai SI, Chen PJ, Lin CT, Boyd DA and Wu TC (2004) Comparison of HPV DNA vaccines employing intracellular targeting strategies. Gene Ther 11:1011-8. doi: 10.1038/sj.gt.3302252
121. Manam S, Ledwith BJ, Barnum AB, Troilo PJ, Pauley CJ, Harper LB, Griffiths TG, 2nd, Niu Z, Denisova L, Follmer TT, Pacchione SJ, Wang Z, Beare CM, Bagdon WJ and Nichols WW (2000) Plasmid DNA vaccines: tissue distribution and effects of DNA sequence, adjuvants and delivery method on integration into host DNA. Intervirology 43:273-81. doi: 53994
122. Nichols WW, Ledwith BJ, Manam SV and Troilo PJ (1995) Potential DNA vaccine integration into host cell genome. Ann N Y Acad Sci 772:30-9.
123. Sorensen SJ, Bailey M, Hansen LH, Kroer N and Wuertz S (2005) Studying plasmid horizontal transfer in situ: a critical review. Nat Rev Microbiol 3:700-10. doi: 10.1038/nrmicro1232
124. Oliveira PH and Mairhofer J (2013) Marker-free plasmids for biotechnological applications - implications and perspectives. Trends Biotechnol 31:539-47. doi: 10.1016/j.tibtech.2013.06.001
125. Williams JA (2014) Improving DNA vaccine performance through vector design. Curr Gene Ther 14:170-89.
126. Kreiss P, Cameron B, Rangara R, Mailhe P, Aguerre-Charriol O, Airiau M, Scherman D, Crouzet J and Pitard B (1999) Plasmid DNA size does not affect the physicochemical properties of lipoplexes but modulates gene transfer efficiency. Nucleic Acids Res 27:3792-8.
127. Yin W, Xiang P and Li Q (2005) Investigations of the effect of DNA size in transient transfection assay using dual luciferase system. Anal Biochem 346:289-94. doi: 10.1016/j.ab.2005.08.029
128. Hornstein BD, Roman D, Arevalo-Soliz LM, Engevik MA and Zechiedrich L (2016) Effects of Circular DNA Length on Transfection Efficiency by Electroporation into HeLa Cells. PLoS One 11:e0167537. doi: 10.1371/journal.pone.0167537
129. Stenler S, Wiklander OP, Badal-Tejedor M, Turunen J, Nordin JZ, Hallengard D, Wahren B, Andaloussi SE, Rutland MW, Smith CE, Lundin KE and Blomberg P (2014) Micro-minicircle Gene Therapy: Implications of Size on Fermentation, Complexation, Shearing Resistance, and Expression. Mol Ther Nucleic Acids 2:e140. doi: 10.1038/mtna.2013.67
130. Lukacs GL, Haggie P, Seksek O, Lechardeur D, Freedman N and Verkman AS (2000) Size-dependent DNA mobility in cytoplasm and nucleus. J Biol Chem 275:1625-9.
131. Marie C, Vandermeulen G, Quiviger M, Richard M, Preat V and Scherman D (2010) pFARs, plasmids free of antibiotic resistance markers, display high-level transgene expression in muscle, skin and tumour cells. J Gene Med 12:323-32. doi: 10.1002/jgm.1441
132. Carnes AE, Luke JM, Vincent JM, Anderson S, Schukar A, Hodgson CP and Williams JA (2010) Critical design criteria for minimal antibiotic-free plasmid vectors necessary to combine robust RNA Pol II and Pol III-mediated eukaryotic expression with high bacterial production yields. J Gene Med 12:818-31. doi: 10.1002/jgm.1499
133. Ribeiro S, Mairhofer J, Madeira C, Diogo MM, Lobato da Silva C, Monteiro G, Grabherr R and Cabral JM (2012) Plasmid DNA size does affect nonviral gene delivery efficiency in stem cells. Cell Reprogram 14:130-7. doi: 10.1089/cell.2011.0093
134. Wang Q, Jiang W, Chen Y, Liu P, Sheng C, Chen S, Zhang H, Pan C, Gao S and Huang W (2013) In Vivo Electroporation of Minicircle DNA as a Novel Method of Vaccine Delivery to Enhance HIV-1-Specific Immune Responses. J Virol. doi: 10.1128/JVI.02757-13
135. Lu J, Zhang F, Xu S, Fire AZ and Kay MA (2012) The extragenic spacer length between the 5' and 3' ends of the transgene expression cassette affects transgene silencing from plasmid-based vectors. Mol Ther 20:2111-9. doi: 10.1038/mt.2012.65

136. Gracey Maniar LE, Maniar JM, Chen ZY, Lu J, Fire AZ and Kay MA (2013) Minicircle DNA vectors achieve sustained expression reflected by active chromatin and transcriptional level. Mol Ther 21:131-8. doi: 10.1038/mt.2012.244
137. Chen ZY, He CY, Ehrhardt A and Kay MA (2003) Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. Mol Ther 8:495-500.
138. Simcikova M, Prather KL, Prazeres DM and Monteiro GA (2015) Towards effective non-viral gene delivery vector. Biotechnol Genet Eng Rev 31:82-107. doi: 10.1080/02648725.2016.1178011
139. Chen ZY, He CY and Kay MA (2005) Improved production and purification of minicircle DNA vector free of plasmid bacterial sequences and capable of persistent transgene expression in vivo. Hum Gene Ther 16:126-31. doi: 10.1089/hum.2005.16.126
140. Nelson J, Rodriguez S, Finlayson N, Williams J and Carnes A (2013) Antibiotic-free production of a herpes simplex virus 2 DNA vaccine in a high yield cGMP process. Hum Vaccin Immunother 9.
141. Lu J, Zhang F and Kay MA (2013) A mini-intronic plasmid (MIP): a novel robust transgene expression vector in vivo and in vitro. Mol Ther 21:954-63. doi: 10.1038/mt.2013.33
142. Williams JA (2014) Replicative Minicircle Vectors with Improved Expression. Nature Technology Corporation, US
143. Pandey S, Kawai T and Akira S (2014) Microbial sensing by Toll-like receptors and intracellular nucleic acid sensors. Cold Spring Harb Perspect Biol 7:a016246. doi: 10.1101/cshperspect.a016246
144. Pavlenko M, Leder C, Moreno S, Levitsky V and Pisa P (2007) Priming of CD8+ T-cell responses after DNA immunization is impaired in TLR9- and MyD88-deficient mice. Vaccine 25:6341-7. doi: 10.1016/j.vaccine.2007.06.016
145. Rottembourg D, Filippi CM, Bresson D, Ehrhardt K, Estes EA, Oldham JE and von Herrath MG (2010) Essential role for TLR9 in prime but not prime-boost plasmid DNA vaccination to activate dendritic cells and protect from lethal viral infection. J Immunol 184:7100-7. doi: 10.4049/jimmunol.0803935
146. Ohlschlager P, Spies E, Alvarez G, Quetting M and Groettrup M (2011) The combination of TLR-9 adjuvantation and electroporation-mediated delivery enhances in vivo antitumor responses after vaccination with HPV-16 E7 encoding DNA. Int J Cancer 128:473-81. doi: 10.1002/ijc.25344
147. Yu YZ, Li N, Ma Y, Wang S, Yu WY and Sun ZW (2013) Three types of human CpG motifs differentially modulate and augment immunogenicity of nonviral and viral replicon DNA vaccines as built-in adjuvants. Eur J Immunol 43:228-39. doi: 10.1002/eji.201242690
148. Arsenault RJ, Kogut MH and He H (2013) Combined CpG and poly I:C stimulation of monocytes results in unique signaling activation not observed with the individual ligands. Cell Signal 25:2246-54. doi: 10.1016/j.cellsig.2013.07.014
149. Gutjahr A, Tiraby G, Perouzel E, Verrier B and Paul S (2016) Triggering Intracellular Receptors for Vaccine Adjuvantation. Trends Immunol 37:573-87. doi: 10.1016/j.it.2016.07.001
150. Wu J and Chen ZJ (2014) Innate immune sensing and signaling of cytosolic nucleic acids. Annu Rev Immunol 32:461-88. doi: 10.1146/annurev-immunol-032713-120156
151. Sugiyama T, Gursel M, Takeshita F, Coban C, Conover J, Kaisho T, Akira S, Klinman DM and Ishii KJ (2005) CpG RNA: identification of novel single-stranded RNA that stimulates human CD14+CD11c+ monocytes. J Immunol 174:2273-9.
152. Wu J, Ma H, Qu Q, Zhou WJ, Luo YP, Thangaraj H, Lowrie DB and Fan XY (2011) Incorporation of immunostimulatory motifs in the transcribed region of a plasmid DNA vaccine enhances Th1 immune responses and therapeutic effect against Mycobacterium tuberculosis in mice. Vaccine 29:7624-30. doi: 10.1016/j.vaccine.2011.08.020
153. Hochheiser K, Klein M, Gottschalk C, Hoss F, Scheu S, Coch C, Hartmann G and Kurts C (2016) Cutting Edge: The RIG-I Ligand 3pRNA Potently Improves CTL Cross-Priming and Facilitates Antiviral Vaccination. J Immunol 196:2439-43. doi: 10.4049/jimmunol.1501958

154. Kulkarni RR, Rasheed MA, Bhaumik SK, Ranjan P, Cao W, Davis C, Marisetti K, Thomas S, Gangappa S, Sambhara S and Murali-Krishna K (2014) Activation of the RIG-I pathway during influenza vaccination enhances the germinal center reaction, promotes T follicular helper cell induction, and provides a dose-sparing effect and protective immunity. J Virol 88:13990-4001. doi: 10.1128/JVI.02273-14

155. Beljanski V, Chiang C, Kirchenbaum GA, Olagnier D, Bloom CE, Wong T, Haddad EK, Trautmann L, Ross TM and Hiscott J (2015) Enhanced Influenza Virus-Like Particle Vaccination with a Structurally Optimized RIG-I Agonist as Adjuvant. J Virol 89:10612-24. doi: 10.1128/JVI.01526-15

156. Zevini A, Olagnier D and Hiscott J (2017) Crosstalk between Cytoplasmic RIG-I and STING Sensing Pathways. Trends Immunol. doi: 10.1016/j.it.2016.12.004

157. Suschak JJ, Wang S, Fitzgerald KA and Lu S (2015) Identification of aim2 as a sensor for DNA vaccines. J Immunol 194:630-6. doi: 10.4049/jimmunol.1402530

158. Suschak JJ, Wang S, Fitzgerald KA and Lu S (2016) A cGAS-Independent STING/IRF7 Pathway Mediates the Immunogenicity of DNA Vaccines. J Immunol 196:310-6. doi: 10.4049/jimmunol.1501836

159. Borggren M, Nielsen J, Bragstad K, Karlsson I, Krog JS, Williams JA and Fomsgaard A (2015) Vector optimization and needle-free intradermal application of a broadly protective polyvalent influenza A DNA vaccine for pigs and humans. Hum Vaccin Immunother 11:1983-90. doi: 10.1080/21645515.2015.1011987

160. Williams JA (2013) Vector design for improved DNA vaccine efficacy, safety and production. Vaccines 1:225-249.

161. Sin JI, Kim JJ, Zhang D and Weiner DB (2001) Modulation of cellular responses by plasmid CD40L: CD40L plasmid vectors enhance antigen-specific helper T cell type 1 CD4+ T cell-mediated protective immunity against herpes simplex virus type 2 in vivo. Hum Gene Ther 12:1091-102. doi: 10.1089/104303401750214302

162. Agadjanyan MG, Chattergoon MA, Holterman MJ, Monzavi-Karbassi B, Kim JJ, Dentchev T, Wilson D, Ayyavoo V, Montaner LJ, Kieber-Emmons T, Sekaly RP and Weiner DB (2003) Costimulatory molecule immune enhancement in a plasmid vaccine model is regulated in part through the Ig constant-like domain of CD80/86. J Immunol 171:4311-9.

163. Kim JJ, Yang JS, Lee DJ, Wilson DM, Nottingham LK, Morrison L, Tsai A, Oh J, Dang K, Dentchev T, Agadjanyan MG, Sin JI, Chalian AA and Weiner DB (2000) Macrophage colony-stimulating factor can modulate immune responses and attract dendritic cells in vivo. Hum Gene Ther 11:305-21. doi: 10.1089/10430340050016049

164. Kim JJ, Tsai A, Nottingham LK, Morrison L, Cunning DM, Oh J, Lee DJ, Dang K, Dentchev T, Chalian AA, Agadjanyan MG and Weiner DB (1999) Intracellular adhesion molecule-1 modulates beta-chemokines and directly costimulates T cells in vivo. J Clin Invest 103:869-77. doi: 10.1172/JCI6024

165. Kim JJ, Yang JS, Montaner L, Lee DJ, Chalian AA and Weiner DB (2000) Coimmunization with IFN-gamma or IL-2, but not IL-13 or IL-4 cDNA can enhance Th1-type DNA vaccine-induced immune responses in vivo. J Interferon Cytokine Res 20:311-9. doi: 10.1089/107999000312450

166. Kim JJ, Trivedi NN, Nottingham LK, Morrison L, Tsai A, Hu Y, Mahalingam S, Dang K, Ahn L, Doyle NK, Wilson DM, Chattergoon MA, Chalian AA, Boyer JD, Agadjanyan MG and Weiner DB (1998) Modulation of amplitude and direction of in vivo immune responses by co-administration of cytokine gene expression cassettes with DNA immunogens. Eur J Immunol 28:1089-103. doi: 10.1002/(SICI)1521-4141(199803)28:03<1089::AID-IMMU1089>3.0.CO;2-L

167. Boyer JD, Nath B, Schumann K, Curley E, Manson K, Kim J and Weiner DB (2002) IL-4 increases Simian immunodeficiency virus replication despite enhanced SIV immune responses in infected rhesus macaques. Int J Parasitol 32:543-50.

168. Sin JI, Kim J, Pachuk C and Weiner DB (2000) Interleukin 7 can enhance antigen-specific cytotoxic-T-lymphocyte and/or Th2-type immune responses in vivo. Clin Diagn Lab Immunol 7:751-8.

169. Kim JJ, Nottingham LK, Sin JI, Tsai A, Morrison L, Oh J, Dang K, Hu Y, Kazahaya K, Bennett M, Dentchev T, Wilson DM, Chalian AA, Boyer JD, Agadjanyan MG and Weiner DB (1998) CD8 positive T cells influence antigen-specific immune responses through the expression of chemokines. J Clin Invest 102:1112-24. doi: 10.1172/JCI3986

170. Sin J, Kim JJ, Pachuk C, Satishchandran C and Weiner DB (2000) DNA vaccines encoding interleukin-8 and RANTES enhance antigen-specific Th1-type CD4(+) T-cell-mediated protective immunity against herpes simplex virus type 2 in vivo. J Virol 74:11173-80.

171. Chattergoon MA, Saulino V, Shames JP, Stein J, Montaner LJ and Weiner DB (2004) Co-immunization with plasmid IL-12 generates a strong T-cell memory response in mice. Vaccine 22:1744-50. doi: 10.1016/j.vaccine.2004.01.036

172. Kutzler MA, Robinson TM, Chattergoon MA, Choo DK, Choo AY, Choe PY, Ramanathan MP, Parkinson R, Kudchodkar S, Tamura Y, Sidhu M, Roopchand V, Kim JJ, Pavlakis GN, Felber BK, Waldmann TA, Boyer JD and Weiner DB (2005) Coimmunization with an optimized IL-15 plasmid results in enhanced function and longevity of CD8 T cells that are partially independent of CD4 T cell help. J Immunol 175:112-23.

173. Kim JJ, Nottingham LK, Tsai A, Lee DJ, Maguire HC, Oh J, Dentchev T, Manson KH, Wyand MS, Agadjanyan MG, Ugen KE and Weiner DB (1999) Antigen-specific humoral and cellular immune responses can be modulated in rhesus macaques through the use of IFN-gamma, IL-12, or IL-18 gene adjuvants. J Med Primatol 28:214-23.

Figure 1: Induction of antigen-specific, adaptive immunity by DNA vaccination. Optimized gene sequences are inserted into a plasmid backbone and then delivered to the host via one of several delivery methods. Vaccine plasmid enters the nucleus of host myocytes and antigen presenting cells by using host cellular machinery. The plasmid components are transcribed and protein is produced. The cell provides endogenous post-translational modifications to antigens, producing native protein conformations. Vaccine-derived endogenous peptides are presented on MHC class I molecules. Engulfment of apoptotic or necrotic cells by APC also allows for cross-presentation of cell-associated exogenous antigens. Secreted antigen is captured and processed by antigen presenting cells, and presented on MHC class II. Antigen experienced APC migrate to the draining lymph node to stimulate $CD4^+$ and $CD8^+$ T cell populations. In addition, shed antigen can be captured by antigen-specific high affinity immunoglobulins on the B cell surface for presentation to $CD4^+$ T cells, driving B cell responses.

Figure 2: Molecular mechanisms of DNA vaccines. Transfected double stranded B DNA (dsDNA) is sensed by cytoplasmic DNA receptors such as interferon-inducible protein 16 (IFI16), DEAD (Asp-Glu-Ala-Asp) box polypeptide 41 (DDX41) and the cGAMP synthase (cGAS), each of which can activate the STING►TBK1►IRF3 pathway to induce type 1 interferon production.[143] An additional cytoplasmic innate immune pathway activated nonspecifically by transfected dsDNA is the cytoplasmic AIM2 inflammasome.[157] Other dsDNA receptors and innate immune activation pathways exist [143], including a recently identified STING/IRF7 signaling pathway required for DNA vaccine immunogenicity.[158] By contrast, the endosomal innate immune receptor TLR9 recognizes specific unmethylated CpG DNA motifs in DNA vaccines. To improve innate immune activation, addition of optimized immunostimulatory CpG

motifs in the vector backbone may be used to increase TLR9 activation. Immunostimulatory RNA expressed from the vector may be utilized to activate alternative RNA sensing innate immune receptors such as RIG-I using an additional RNA Polymerase III RNA expression cassette [117] (plasmid backbone adjuvant) or incorporation of RNA recognizing TLR agonist motifs such as CpG RNA into the 3' UTR.[152] Due to limited transgene expression after DNA vaccination in large animals, vector modifications (*e.g.* <500 bp bacterial region Nanoplasmid™ vectors; intronic bacterial region MIP vectors) and deliveries (*e.g.* Electroporation) that improve transgene expression also improve adaptive immunity.[62, 125, 159] Adapted under a Creative Commons Attribution license from Williams, 2013.[160]

943 **Table 1: Molecular adjuvants tested *in vivo*.**

Molecular Adjuvant	Molecule Type	Animal Model	Adaptive Response Effect	References
CD40L	Co-Stimulatory	Mice	Cellular	[161]
CD80/86	Co-Stimulatory	Mice, NHP	Cellular	[162]
GM-CSF	Cytokine	Mice	Humoral	[163]
ICAM-1	Co-Stimulatory	Mice	Cellular	[164]
IFN-γ	Cytokine	Mice, NHP	Cellular	[165]
IL-2	Cytokine	Mice	Cellular, Humoral	[165, 166]
IL-4	Cytokine	Mice, NHP	Humoral	[166, 167]
IL-7	Cytokine	Mice	Cellular, Humoral	[168]
IL-8	Chemokine	Mice	Cellular, Humoral	[169, 170]
IL-10	Cytokine	Mice	Cellular	[166]
IL-12	Cytokine	Mice, NHP	Cellular	[98, 171]
IL-15	Cytokine	Mice, NHP	Cytokine	[98, 172]
IL-18	Cytokine	Mice, NHP	Cytokine	[166, 173]
MCP-1	Chemokine	Mice	Humoral	[169]
M-CSF	Cytokine	Mice	Cellular	[163]
MIP-1α	Chemokine	Mice	Humoral	[169]
RANTES	Chemokine	Mice	Cellular	[169, 170]

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Figure 1

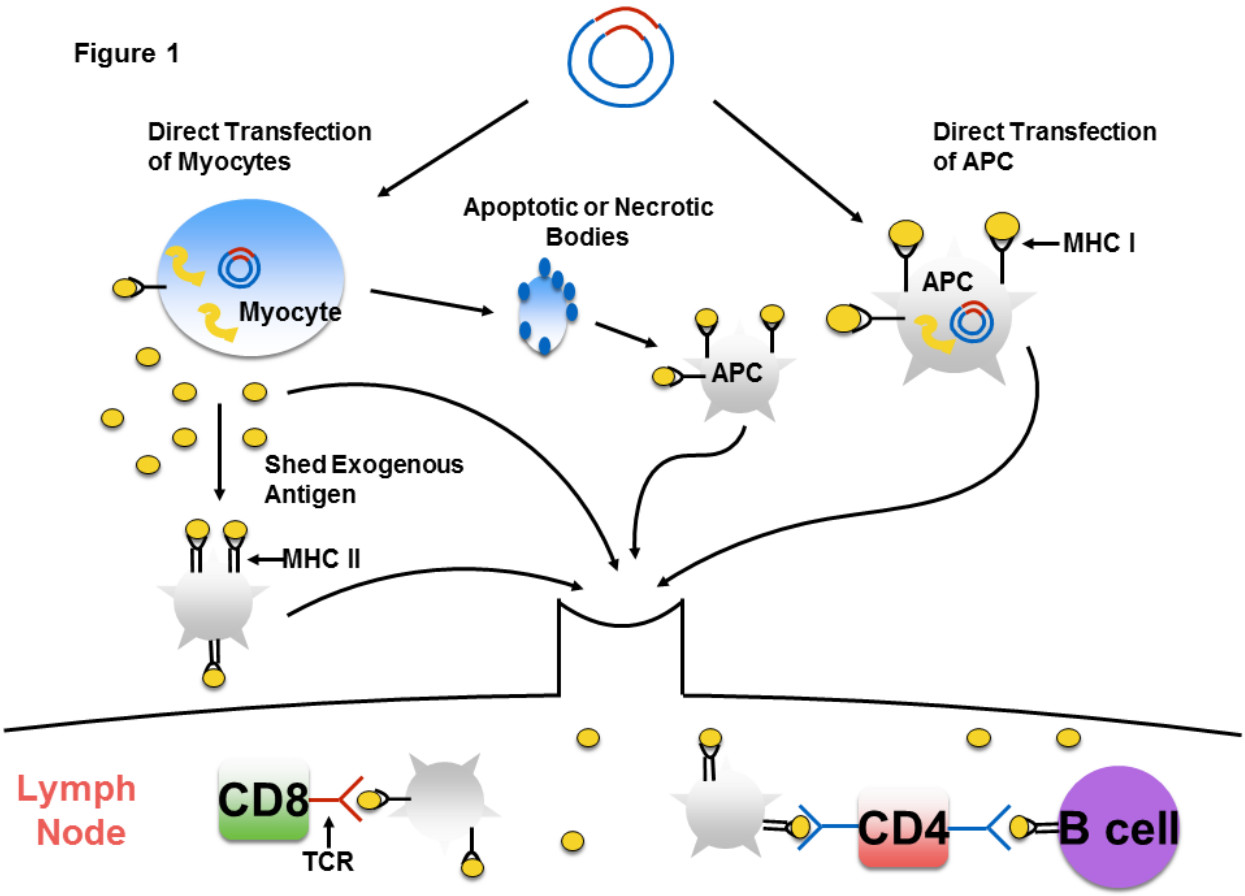
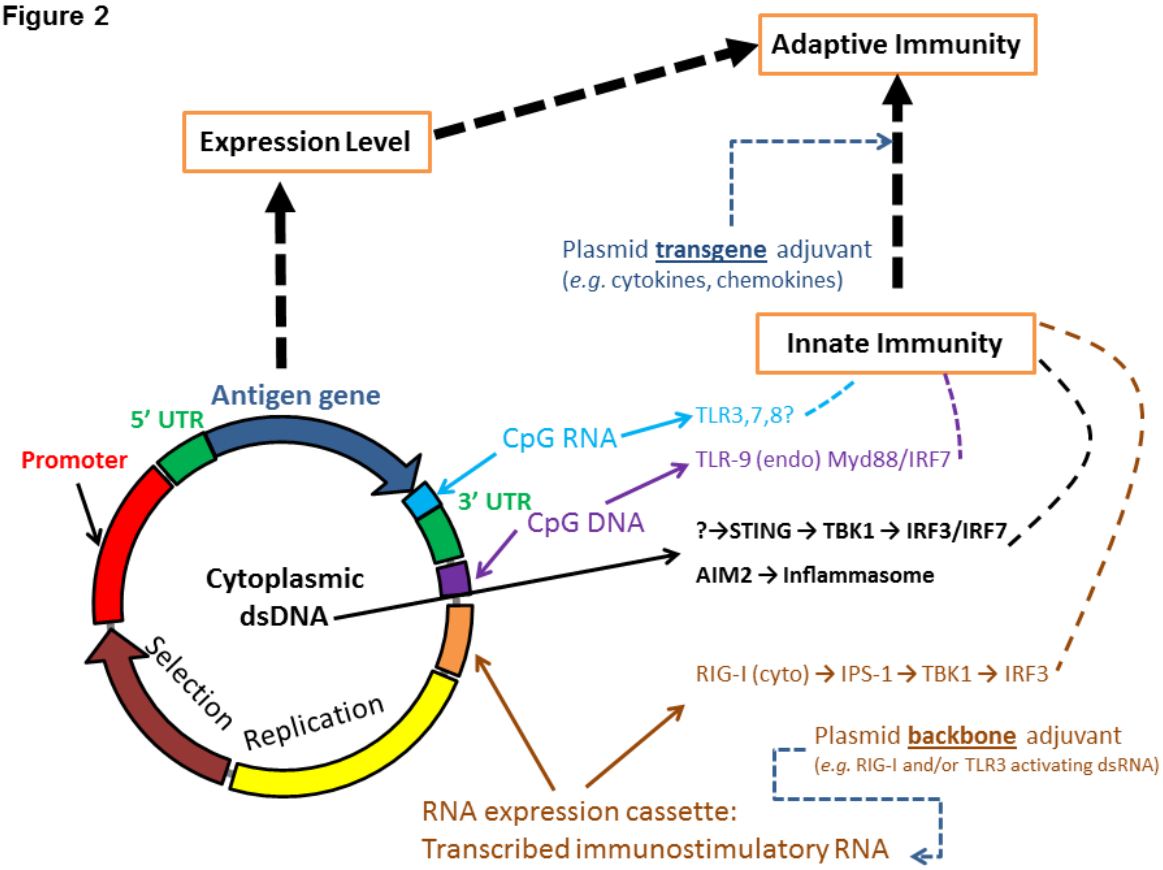

Direct Transfection of Myocytes
Apoptotic or Necrotic Bodies
Direct Transfection of APC
Myocyte
APC
MHC I
APC
Shed Exogenous Antigen
MHC II
Lymph Node
CD8
TCR
CD4
B cell

Figure 2


Adaptive Immunity
Expression Level
Plasmid transgene adjuvant
(e.g. cytokines, chemokines)
Innate Immunity
Antigen gene
5' UTR
Promoter
3' UTR
CpG RNA
TLR3,7,8?
TLR-9 (endo) Myd88/IRF7
CpG DNA
?→STING → TBK1 → IRF3/IRF7
AIM2 → Inflammasome
Cytoplasmic
dsDNA
Selection
Replication
RIG-I (cyto) → IPS-1 → TBK1 → IRF3
Plasmid backbone adjuvant
(e.g. RIG-I and/or TLR3 activating dsRNA)
RNA expression cassette:
Transcribed immunostimulatory RNA